

METHODS AND COMPOSITIONS FOR DIAGNOSING HEPATOCELLULAR CARCINOMA

This application claims the benefit of U.S. Provisional Application Serial No. 60/393,982 filed on July 3, 2002, hereby incorporated in its entirety by reference.

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with Government support under Grant No. U19A148214 from the National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND

The field of the invention is the diagnosis of hepatocellular carcinoma (HCC).

Hepatocellular carcinoma (HCC) is the most prevalent form of liver cancer worldwide. Incidence of the disease varies geographically from between 1 in 5,000 in Asia to 1 in 20,000 in western nations (Wildi et al., 2002). Patients with chronic liver disease are at increased risk for development of hepatocellular carcinoma. This is particularly true for individuals with liver cirrhosis who should be closely monitored for development of this disease.

Currently, it is difficult to diagnose HCC. Methods employed generally rely on imaging techniques such as MRI, CT, and ultrasound and are of little use in detecting the disease in its earliest stages. As with most cancers, early detection of HCC would leave physicians with more treatment options and patients with a better prognosis (Befeler and Bisceglie, 2002).

Better imaging reagents would enhance the sensitivity and broaden the applicability of currently used scanning methodologies. Proteins expressed specifically or preferentially on the surface of HCC cells could be targeted by an antibody or other targeting reagent that is conjugated to an imaging agent. Such conjugates would aid in diagnosis of the disease at an early stage.

The literature describes a few serodiagnostic markers indicative of HCC, including alpha-fetoprotein (AFP), *Lens culinaris* agglutinin-reactive fraction (AFP-L3), and des-gamma-carboxy prothrombin or PIVKA-II (Shimizu et al., 2002; Ikoma et al., 2002; Fujiyama et al., 1986; Naraki et al., 2002). Unfortunately, at best, elevated levels of these serum proteins are detected in only about 50% of HCC patients. A significant increase in the sensitivity of HCC diagnosis can be achieved by combining tests for

AFP, AFP-L3 and PIVKA-II. However, even when all three tests are combined, the sensitivity is only about 87% (Fujiyama et al., 2002).

Identification of new serodiagnostic markers specific to HCC and present in a large percentage of HCC patients would greatly improve the diagnosis of this disease and be more cost effective than commonly used scanning methodologies and/or the combined use of all currently available serodiagnostic assays.

These and other limitations and problems of the past are solved by the present invention.

BRIEF SUMMARY OF THE INVENTION

The present invention relates to the detection of hepatocellular carcinoma (HCC) by assaying patient samples such as tissue, plasma, serum, etc. for the presence and level of specific HCC related proteins. Some of these proteins will be cell associated, while others will not be cell associated. A finding of elevated levels of one or more of these proteins in a patient sample indicates that the patient has hepatocellular carcinoma. HCC diagnosis based on quantification of the HCC related protein(s) will be dependent upon research that will define a variety of parameters. These parameters will include: (a) a determination of the relative levels of the HCC related proteins in diseased versus normal patient samples (as an example of a control level, but not limited to), and (b) the specificity, sensitivity and reproducibility of the assay or assays employed.

The present invention also relates to identification of tumor markers that may be targeted by specific reagents to enhance early diagnosis of HCC by traditional scanning methodologies. Proteins expressed specifically on the surface of HCC cells could be targeted by an antibody or other targeting reagent (e.g. soluble receptor or ligand) that binds specifically to the cell-associated HCC protein. The targeting moiety is conjugated to an imaging agent to enable visualization of the construct.

The proteins that are useful in accordance with the present invention are: phospholipase A2 (Group XIII) (SEQ ID Nos. 1-2); phospholipase A2 (group VII) (SEQ ID No. 12); anti-thrombin III (SEQ ID No. 3); apolipoprotein B (SEQ ID No. 4); group C specific vitamin D binding protein (SEQ ID Nos. 5-6); gamma-glutamyl hydrolase (SEQ ID No. 7); nicastrin (SEQ ID No. 8); pregnancy associated plasma protein A, plasma glutamate carboxypeptidase (SEQ ID No. 11); secretory carrier membrane protein-3 (SEQ ID Nos. 9-10); and other hypothetical proteins described herein. Not all of the

proteins that are useful within the methods of the present invention are found exclusively in HCC patients. Some proteins will be found in both patients with and without HCC. In these cases, HCC affected individuals will be distinguished from non diseased individuals by a significant elevation in the amount of one or more of the proteins described in the current invention.

The invention will best be understood by reference to the following detailed description of the preferred embodiment. The discussion below is descriptive, illustrative and exemplary and is not to be taken as limiting the scope defined by any appended claims.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Expression microarray analysis of tumor samples from Hepatitis C (HCV) infected patients with hepatocellular carcinoma (HCC) led to the identification of genes that were specifically up-regulated in hepatocellular carcinoma tumor tissue when compared to HCV infected cirrhotic non-tumor tissue, and normal liver.

Liver and HCC samples were obtained during surgical procedures with prior informed consent from all persons involved. HCC samples included 21 from HCV infected patients and 1 from a patient infected with Hepatitis B. In addition, 4 samples of normal, non-diseased liver and 8 samples of HCV infected, cirrhotic liver with no evidence of HCC were used for analysis.

Total RNA was isolated as described in Geiss et al. (2001). RNA amplification was performed using a T7 RNA polymerase protocol (Eberwine, 1996) with the AmpliScribe Transcription kit (Epicentre Technologies, Madison, Wisconsin) as described by the manufacturer. The quality of amplified RNA samples was evaluated using capillary electrophoresis in an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California).

cDNA microarrays were constructed by the University of Washington's Center for Expression Array Technology using PCR products generated by amplification of sequence verified I.M.A.G.E. consortium clones obtained from Research Genetics (St. Louis, MO) (Lennon et al. 1996). Microarrays were constructed as previously described (Geiss et al. 2001). A human high density set consisted of two arrays, each of which represented 7,296 human clones in duplicate with a number of additional control sequences, for a total of 14,976 clones (approximately 13,597 unique I.M.A.G.E. cDNA clones). Each single experiment involved interrogation of two slides for which the dye

labels had been reversed (fluor reversal methodology as described in Geiss et al., 2000; Geiss et al., 2001). A total of at least four separate hybridization measurements were taken per gene per experiment.

5 Protocols for probe synthesis, microarray hybridization, and wash conditions are as previously described (Geiss et al. 2001). Microarrays were scanned and the images were quantified using a custom spot-finding program, Spot-On Image (Geiss et al, 2000 and Geiss et al., 2001), that calculated the standard deviations and the mean ratios between the expression levels of each gene in the analyzed pair of samples. Raw data and sample information were entered into a custom designed database, Expression
10 Array Manager, and evaluated using Rosetta Biosoftware's Resolver® Version 3.0 (Rosetta Biosoftware, Kirkland, WA), a software package for the storage and analysis of microarray expression data. This package implements common statistical procedures (clustering, trend analysis, similarity searches based on a BLAST-related algorithm, etc.) together with a sophisticated error model to compensate for biological
15 and experimental variation.

 The expression microarray data was processed by two different methods. The first involved examining only HCV-infected HCC patient samples and sorting for genes that were significantly ($p < 0.01$) up-regulated more than two-fold in tumor versus non-tumor liver samples from the same patient. Genes that met these criteria in ten or more
20 patients were then analyzed relative to samples from HCV infected patients with liver cirrhosis but no tumors and also relative to samples of normal healthy liver. If the gene was unchanged or down-regulated in these control samples, its potential for use as a diagnostic target was further evaluated using information available in the National Center for Biotechnology Information databases (Unigene, OMIM, LocusLink, and
25 HomoloGene) and currently published literature regarding the location and function of its protein product. The protein products of the genes that meet the above criteria and are (a) secreted or likely to be present on the plasma membrane and are (b) noted to be preferentially or specifically expressed in liver, are likely to be diagnostic indicators of HCC. The following are an example of some of these proteins while their
30 corresponding amino acid sequences and variants thereof are included in the sequence listing accompanying this application:

PGLA2G13 (phospholipase A2 Group XIII; IMAGE EST: 297107; GenBank AF349540; Unigene: 333175; mRNA: NM 032562; protein: NP 115951; (SEQ ID Nos. 1-2));

SERPINC1 (serine or cysteine proteinase inhibitor; anti thrombin III; IMAGE EST:85643; GenBank X68793; Unigene: Hs.75599; mRNA: 000488; protein: NP 000479; (SEQ ID No. 3));

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APOB (apclipoprotein B; IMAGE EST: 206632; GenBank X04506; Unigene: Hs.585; mRNA: NM 000384; protein: NP 000375; (SEQ ID No. 4));

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GC (group C specific vitamin D binding protein; IMAGE EST: 195340; GenBank M12654; Unigene: Hs.198246; mRNA: NM 000583; protein: NP 000574; (SEQ ID Nos. 5-6));

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GGH (gamma-glutamyl hydrolase; conjugase; folylpolygammaglutamyl hydrolase; IMAGE EST: 809588; GenBank U55206; Unigene: Hs.78619; mRNA: NM 003878; protein: NP 003869; (SEQ ID No. 7)); and

NCSTN (nicastrin; IMAGE EST: 199645; GenBank R96527; Unigene: Hs.4788; (SEQ ID No. 8)).

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The function of a number of genes that were up-regulated in the HCC samples but not in control samples is unknown. Included herein are the protein products of these genes and their use as diagnostic markers for HCC. These gene products are as follows:

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Protein coded by the gene specified as: IMAGE EST: 241475; GenBank H90421; Unigene: Hs.41407;

Protein coded by the gene specified as: IMAGE EST: 293094; GenBank N91620; Unigene: Hs.12160;

Protein coded by the gene specified v: IMAGE EST: 430221; GenBank AA010360; Unigene: Hs.60380;

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Protein coded by the gene specified as: IMAGE EST: 52990; GenBank R15441; Unigene: Hs.4774;

Protein coded by gene specified as: IMAGE EST: 153779; GenBank R48248; Unigene: Hs.183171; mRNA: NM 024838; protein: NP 079114 hypothetical protein FLJ22002;

(SEQ ID No. 13).

The second method of processing the microarray data yielded similar results.

Error probabilities were used to filter the initial 13,597 member gene set to a set of 2302 genes that demonstrated differential regulation of two-fold or greater with 95%

5 confidence ($p \leq 0.05$) in at least 4 out of 20 experiments involving the comparison of HCC tumor versus matched non-tumor tissues. A keyword search was then applied to this group to identify genes encoding putative secreted and/or plasma membrane proteins. The resultant small gene subset was manually filtered to exclude those genes that were down-regulated in most tumors. Finally, a set of 11 genes was selected and
10 used for two dimensional clustering analyses of all 4 experiments. Four out of 11 genes showed a pronounced up-regulation of gene expression in about 60 to 70% of all tumor versus non-tumor liver experiments. Also, all four genes were significantly up-regulated in experiments involving pooled tumor versus normal liver samples. The four gene products are listed below and include several of the proteins noted above.

15 Corresponding amino acid sequences and variants thereof are listed in the sequence listing accompanying this patent.

SCAMP3 (secretory carrier membrane protein-3; IMAGE EST: 156045; GenBank R72518, Unigene: Hs.200600; mRNA: NM 005698; protein: NP 005689; (SEQ ID Nos. 9-10));

20 PGCP (plasma glutamate carboxypeptidase; IMAGE EST: 796263; Unigene: Hs.197335; (SEQ ID No. 11)) Gingras et al. 1999;

PGLA2G13 (phospholipase A2 Group XIII; IMAGE EST: 297107; GenBank AF349540; Unigene: 333175; mRNA: NM 032562; protein: NP 115951; (SEQ ID Nos. 1-2)); and

PLA2G7 (phospholipase A2 group VII; IMAGE EST: 238821; GenBank H65029;

25 Unigene: Hs.93304; mRNA: NM 005084; protein: NP 005075; (SEQ ID No. 12)).

Several of the proteins that were identified by either method will find use for the diagnosis of HCC. An elevated level of one or more of these proteins in a patient sample is indicative of disease. Diagnostic proteins are expressed in either a cell associated or non-cell associated way. The method of diagnosis will depend on
30 whether the diagnostic or predictive protein is cell associated or non-cell associated.

The non-cell associated proteins include PGCP (SEQ ID No. 11), PGLA2G13 (SEQ ID Nos. 1-2), PLA2G7 (SEQ ID No. 12), SERPINC1 (SEQ ID No. 3), APOB (SEQ ID No. 4), GC (SEQ ID Nos. 5-6), and GGH (SEQ ID No. 7). The diagnosis of HCC

may result from quantification of these proteins individually or in combination in patient samples such as blood, plasma, serum, urine, etc.

The presence and quantity of non-cell associated proteins within a patient sample will be measured by state of the art techniques which include, but are not limited to, ELISA, sandwich ELISA, radiolabeled immunoassay (RIA) or other competitive binding assay that is based on the use of specific antibodies. Alternatively, activity assays for quantification of those non-cell associated proteins that are enzymes (PGCP (SEQ ID No. 11); PLA2G7 (SEQ ID No. 12); PLA2G13 (SEQ ID Nos. 1-2); SERPINC1 (SEQ ID No. 3); and GGH (SEQ ID No. 7)) may also be employed.

In addition or in the alternative, HCC may be diagnosed by imaging or scanning methodologies employing targeting agent-imaging agent conjugates. Preferred proteins for this aspect of the present invention are the cell associated proteins, SCAMP3 (SEQ ID Nos. 9-10) and NCSTN (SEQ ID No. 8), and will find use as imaging targets when used in combination with labeling and scanning technologies.

The targeting agents useful in the practice of the present invention include, but are not limited to, antibodies or soluble receptors or ligands or other agents that specifically bind proteins expressed by HCC cells. When conjugated to imaging agents, these targeting agents enable visualization of tumor cells.

The imaging agents useful in the practice of the present invention include, but are not limited to, radioisotopes, electron dense dyes and/or a variety of other reagents visible to scanning technologies that have been well described in the literature (see for example: Vera et al. 1995; Shen et al. 1996; Matsumura et al. 1994; Reimer et al. 1994; Koral et al. 1994; Winzelberg et al. 1992; Perkins et al. 1993).

The targeting molecule-imaging agent-conjugate will be administered to the patient intravenously prior to employment of the imaging application thereby enabling and/or enhancing tumor visualization. The molecular imaging agent-conjugate may bind to the cell associated HCC related protein or may be subject to receptor mediated uptake where the receptor is the cell associated HCC related protein.

Other methods of the present invention involve the use of liver tissue samples. For these aspects of the present invention, the patient sample may be obtained by biopsy or other technique known in the art.

An embodiment of the present invention useful in the analysis of tissue samples includes employing immunocytochemistry or immunohistochemistry techniques using a

cell-associated HCC related protein specific antibody conjugated to imaging agents.

In addition, tissue samples may be evaluated by assaying for transcription of one or more of the cell-associated or non-cell associated HCC related proteins by RT-PCR or nucleic acid hybridization methods.

5 The diagnosis of HCC may result from quantification of these proteins individually or in combination using any of the methods noted above.

Of direct relevance herein are the development of polyclonal antibodies which bind to recombinant human PLA2G13 (SEQ ID Nos. 1-2) and the use of said antibodies in quantification or visualization of PLA2G13 (SEQ ID Nos. 1-2). The generation of
10 polyclonal antisera by immunization of rabbits and the use of Western Blot analysis, as outlined below, will be familiar to one skilled in the art.

Polyclonal antibodies were generated by immunizing rabbits with either the recombinant human PLA2G13 (variant 1; SEQ ID No. 1) or with synthetic peptides (SEQ ID Nos. 14-16) representing portions of human PLA2G13 (SEQ ID No. 1) coupled
15 to a carrier protein. The sequence of each of these peptides is indicated below with an additional cysteine residue added to the 5'-terminus of peptide #1 as a means of conjugation to the carrier protein.

(SEQ ID No. 14) Peptide #1: 5' CSDTSPDTEESYSD 3'

(SEQ ID No. 15) Peptide #2: 5' CSDLKRS LGFVSKVE 3'

20 (SEQ ID No. 16) Peptide #3: 5' CAEEEEKEEL 3'

Antisera from rabbits immunized with recombinant human PLA2G13 (SEQ ID No. 1) or with carrier protein conjugates of peptides #1 or #3 contained antibodies that bound recombinant human PLA2G13 (SEQ ID No. 1). This was verified by a Western Blot Assay.

25 The recombinant human PLA2G13 (SEQ ID No. 1) used in Western Blot Assay was expressed in, and purified from E. coli using known molecular biological and biochemical methods as outlined in Koduri et al. (2002) for a similar protein. Additionally, the recombinant human PLA2G13 (SEQ ID No. 1) was refolded as and characterized as described for a similar protein by Valentin et al., 1999, indicating that it
30 is in its native conformation. Polyclonal antibodies that bind the recombinant human PLA2G13 (SEQ ID No. 1) in a native conformation will likely bind endogenous or native PLA2G13 (SEQ ID No. 1-2) in humans or human derived material. The generation of polyclonal antibodies that bind PLA2G13 (SEQ ID No. 1) enables the development of

antibody based assays to detect endogenous PLA2G13 (SEQ ID Nos. 1-2) in patients or detect and quantify PLA2G13 (SEQ ID Nos. 1-2) in patient derived material. Additionally, the anti-PLA2G13 (SEQ ID No. 1) antibodies can serve as the targeting portion of imaging conjugate(s).

5 The discussion above is descriptive, illustrative and exemplary and is not to be taken as limiting the scope defined by any appended claims.

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